Applicant(s) :   Yih-Lin Chung
Serial No.   :   10/798,119
Filed   :   March 11, 2004
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Attorney Docket No.: 55701-004002
Client Ref. No.: 0668-A20348US

REMARKS

The present document is submitted in response to the final Office Action dated October 1, 2007 ("Office Action").

Applicant has rephrased previously presented claim 1 to promote clarity, and fixed a minor deficiency in claim 11. No new matter has been introduced. **The amendments should be entered as they raise no new issues that will require further consideration or search and also do not touch the merits of the application within the meaning of 37 C.F.R. § 1.116(b).**

Claims 1, 5, 11, and 14-17 are under examination. Applicant respectfully requests that the Examiner reconsider the application as amended in view of the following remarks.

Rejection under U.S.C. § 112, First Paragraph (New Matter)

The Examiner rejects claims 1, 5, 11, and 14-17 for including new matter. See the Office Action, pages 3-4. More specifically, she asserts that the specification provides no support for Applicant's amendment to previously presented claim 1 submitted in their last reply to define the term "therapeutic gain" recited therein. See the Office Action, page 3, second paragraph. Applicant respectfully disagrees. Note that Applicant has now further amended claim 1 to promote clarity. The definition of "therapeutic gain" recited in currently amended claim 1 is substantially the same as that recited in previously presented claim 1. In other words, the Examiner's ground for rejection is applicable to currently amended claim 1. Applicant thus traverses below this rejection as applied to currently amended claim 1.

Currently amended claim 1 covers a method for **increasing therapeutic gain** in chemotherapy or radiotherapy. This claim defines the term "therapeutic gain" as "(i) preventing complications or sequelae of a disorder, both of which are induced by radiation or chemotherapy ... ; (ii) protecting normal tissues from cell death; or (iii) promoting radiation-induced wound healing in mucositis and dermatitis." The disorder includes mucositis, dermatitis, ulceration, tissue necrosis, fibrosis, xerostomia, plantar-palmar syndrome, and tumorigenesis.

The preamble of currently amended claim 1, i.e., for increasing **therapeutic gain,**" indicates that the **purpose** of the claimed method is to achieve **therapeutic gain.** In other words, the definition of **"therapeutic gain"** recited in currently amended claim 1 sets forth the **purpose** of the claimed method. Note that this definition is essentially the same as the disclosure in the specification regarding the **purpose** of the claimed method. See page 3, lines 6-22.[1] Thus, Applicant submits that the instant specification fully supports the definition of therapeutic gain recited in currently amended claim 1.

Rejection under U.S.C. § 112, First Paragraph (Enablement)

Claims 1, 5, 11, and 14-17 are rejected for lack of enablement. See the Office Action, pages 3-4. The Examiner asserts that "[a]lthough the applicant has provided some working examples to support increased therapeutic gain in radiotherapy based on *tumor control*, the applicant has failed to disclose *any* examples or data to support prevention of the *same [tumor]* as a result of treatment with a hyperacetylating agent," and that "the status in the art regarding prevention of *cancer* in general is so uncertain." See the Office Action, page 4, third and fourth paragraphs; emphasis added. Clearly, the Examiner views the methods covered by the rejected claims as directed to prevention of cancer/tumor. Applicant would like to point out that this is a misunderstanding.

Claim 1, the only independent claim, covers a method for increasing **therapeutic gain** by administering to a subject in need a composition containing a histone hyperacetylating agent and a pharmaceutically acceptable carrier. This claim defines therapeutic gain as (i) preventing complications or sequelae of a disease, both of which are induced by radiation or chemotherapy, (ii) protecting normal tissues from cell death, and (iii) promoting radiation-induced wound healing in mucositis and dermatitis. As the medical conditions included in this definition are all **side effects** associated with

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[1] The specification states that "[t]he **purpose** of the method and pharmaceutical composition of the present invention includes... preventing the onset of or ameliorating the radiation- and/or chemotherapy-induced complications or sequelae of mucositis, dermatitis, ulceration, fibrosis, xerostomia, plantar-palmar syndrome, and tumorigenesis" (see page 3, lines 6-13; emphasis added); and that the composition "promoted radiation-induced wound healing in mucositis and dermatitis" and "reduced radiation-induced normal tissue fibrosis" (see page 3, lines 17-19). The definition of therapeutic gain recited in claim 1 is based on the above quoted passages.

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chemotherapy or radiotherapy, a skilled person in the art would readily understand that the **therapeutic gain** refers to treating these **side effects**. In other words, claim 1, aiming at treating certain **side effects** induced by radiotherapy or chemotherapy, is not directed to **cancer/tumor prevention** per se, as incorrectly asserted by the Examiner. The Examiner's ground for rejection, based on a misunderstanding of fact, is clearly invalid.

Indeed, the specification provides a number of examples showing the efficacy of histone deacetylase inhibitors (histone hyperacetylating agents) in treating the chemotherapy- or radiotherapy-induced side effects as recited in claim 1:

- Example 3 shows that histone deacetylase inhibitors reduced **acute radiation-induced normal tissue damage**;

- Example 4 demonstrates that histone deacetylase inhibitors suppressed **radiation-induced skin damage,** e.g., acute dermatitis and desquamation;

- Example 8 shows that histone deacetylase inhibitors prevented late **radiation-induced skin tumor formation**;

- Example 10 indicates that histone deacetylase inhibitors ameliorated **radiation-induced normal tissue damage**; and

- Example 13 demonstrates that histone deacetylase inhibitors were effective in treating **radiation-induced mucositis.**

Further, Chung et al., an article co-authored by the inventor himself, also teaches that histone deacetylase inhibitors suppressed **cutaneous syndrome induced by radiation** (copy attached as Exhibit 1).[2]

Based on these teachings, a skilled artisan would readily recognize the efficacy of histone hyperacetylating agents (e.g., histone deacetylase inhibitors) in treating radio/chemotherapy-induced **side effects**, e.g., normal tissue damage, dermatitis, mucositis. In other words, he or she would know that the method of claim 1, i.e., applying histone hyperacetylating agents to a subject, is effective in improving certain **side effects** induced by radio/chemotherapy (increasing **therapeutic gain**).

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[2] This article, published (12/15/2003) within a year from the filing date of the present application (3/11/2004), discloses Inventor Yih-Lin Chung's own work. Thus, it is not citable against the present application.

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In view of the above remarks, Applicant submits that the present specification fully enables claim 1. The Examiner rejects claims 5, 11, and 14-17, all of which depend from claim 1, on the same ground. For the same reasons, these claims are also enabled.

It is respectfully requested that the Examiner withdraw this rejection.

Rejection under U.S.C. § 112, Second Paragraph

Claims 1, 5, 11, and 14-17 are rejected as indefinite. More specifically, the Examiner holds that, the clause "therapeutic gain comprises **preventing** radiation or chemotherapy-induced complications or sequelae of ... ," recited in claim 1 is not clear as one would not be certain what the term **"preventing"** modifies. See the Office Action, page 5, second paragraph.

For the sole purpose of facilitating prosecution, Applicant has amended claim 1 to make it clear that "preventing" modifies "complications or sequelae of a disease, both of which are induced by radiation or chemotherapy." This amendment is believed to have overcome the rejection.

Rejection under U.S.C. § 103

The Examiner rejects claims 1, 5, 11, 14-17 for obviousness on two grounds, which are traversed separately below.

First, claims 1, 5, 11, and 14-16 are rejected as obvious over Samid, US Patent No. 5,877,213 ("Samid"). Applicant respectfully disagrees.

As pointed out above, claim 1 is drawn to a method of administering to a **subject in need** a histone hyperacetylating agent to increase therapeutic gain as defined in this claim, i.e., treating certain side effects induced by chemotherapy or radiotherapy. Clearly, the **"subject in need"** recited in claim 1 refers to a subject who needs treatment of the **side effects** caused by chemotherapy or radiotherapy. In other words, the claimed method aims at treating **a subject having such a need** with a histone hyperacetylating agent.

Samid teaches a method of treating **anemia, cancer, AIDS,** or severe β**-chain hemoglobinopathies** by administering to a subject phenylacetate, a histone

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hyperacetylating agent. The Samid method is applied only to subjects who are suffering from these **diseases**, and not to those suffering from the chemotherapy or radiotherapy induced **side effects**, as recited in claim 1. Claim 1 therefore is not rendered obvious by Samid. Nor does this reference render obvious claims 5, 11, and 14-16, all of which depend from claim 1.

Second, claims 1 and 17 are rejected as obvious over Samid in view of Shufeng et al. ('Shufeng'). See the Office Action, page 7.

As discussed above, claim 1 covers a method of administering a histone hyperacetylating agent to a subject suffering from certain **side effect**s induced by chemotherapy or radiotherapy; Samid discloses a method of administering a histone deacetylase inhibitor to a patient suffering from **cancer**.

Shufeng teaches using 5,6-dimethylxanthenone-4-acetic acid (DMXAA, a histone hyperacetylating agent) for treating **cancer**. In other words, this reference teaches using a histone hyperacetylating agent to a patient suffering from **cancer**, and not a patient suffering from the **side effects** recited in claim 1.

In sum, neither Samid nor Shufeng suggests administering a histone hyperacetylating agent to a subject suffering from the chemo/radiotherapy-induced side effects, as required by the method of claim 1. Applicant thus submits that these two references, either taken alone or in combination, do not render obvious claim 1. Neither do they render obvious claim 17, being dependent from claim 1.

CONCLUSION

It is believed that all of the pending claims have been addressed. However, the absence of a reply to a specific rejection, issue or comment does not signify agreement with or concession of that rejection, issue or comment. In addition, because the arguments made above may not be exhaustive, there may be reasons for patentability of any or all pending claims (or other claims) that have not been expressed. Finally, nothing in this paper should be construed as an intent to concede any issue with regard to any claim, except as specifically stated in this paper, and the amendment of any claim does not necessarily signify concession of unpatentability of the claim prior to its amendment.

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No fee is believed to be due. Please apply any other charges or credits to Deposit Account No. 50-4189, referencing Attorney Docket No. 55701-004002.

Respectfully submitted,

Date: _____12/17/07_____

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Antitumor histone deacetylase inhibitors suppress cutaneous radiation syndrome: Implications for increasing therapeutic gain in cancer radiotherapy

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Abstract

Radiotherapy is an effective treatment for head and neck, skin, anogenital, and breast cancers. However, radiation-induced skin morbidity limits the therapeutic benefits. A low-toxicity approach to selectively reduce skin morbidity without compromising tumor killing by radiotherapy is needed. We found that the antitumor agents known as histone deacetylase (HDAC) inhibitors (phenylbutyrate, trichostatin A, and valproic acid) could suppress cutaneous radiation syndrome. The effects of HDAC inhibitors in promoting the healing of wounds caused by radiation and in decreasing later skin fibrosis and tumorigenesis were correlated with suppression of the aberrant expression of radiation-induced transforming growth factor β and tumor necrosis factor α. Our findings implicate that the inhibition of HDAC may provide a novel strategy to increase the therapeutic gain in cancer radiotherapy by not only inhibiting tumor growth but also protecting normal tissues. [Mol Cancer Ther. 2004;3(3):317–325]

Introduction

Radiotherapy is an effective treatment for head and neck, breast, skin, and anogenital cancers. However, its therapeutic benefit is limited by radiation-induced skin injuries or cutaneous radiation syndrome (CRS), which includes acute reactions of swelling, dermatitis, desquamation, and ulceration, and long-term effects of fibrosis, necrosis, and the development of life-threatening sequelae of sarcoma, squamous, and basal cell carcinoma (1, 2). In fact, the skin is affected in every form of the external radiotherapy of internal organs. The topical application of steroidal or nonsteroidal anti-inflammatories is the most common

treatment for CRS, yet the results are unsatisfactory. An approach to selectively reduce skin morbidity without compromising the tumor-killing effects of radiotherapy is a long-sought goal in radiation oncology.

After radiation injury, the release of cytokines [such as tumor necrosis factor (TNF-α)] and growth factors [such as transforming growth factor (TGF-β)] in irradiated tissues perpetuates and augments the inflammatory response, while promoting fibroblast recruitment and proliferation but inhibiting epithelial cell growth (3–10). The amplified injury response to radiation by the persistent secretion of TNF-α and TGF-β from epithelial, endothelial, and connective tissue cells, which is possibly caused by a modification in the genetic programming of cell differentiation and proliferation, leads to the histological modifications that characterize CRS (11–14). The chronic activation of TGF-β pathway also stimulates late tumorigenesis (15, 16). Thus, CRS and radiation-induced carcinogenesis could be regarded as a genetic disorder of the wound healing process after radiation exposure. This prompted us to propose whether there is a gene modulator that can simultaneously both reverse the skewed expression of TNF-α and TGF-β in irradiated skin and modulate the oncogenes or tumor suppressors in tumor cells.

A class compound of gene modulators, histone deacetylase (HDAC) inhibitors, such as short-chain fatty acids (phenylbutyrate and valproic acid) and hydroxamic acids (trichostatin A), activates and represses a subset of genes by remodeling the chromatin structure via the altered status in histone acetylation (17, 18). Histone hyperacetylation results in the up-regulation of cell-cycle inhibitors (p21[Cip1], p27[Kip1], and p16[INK4]), the down-regulation of oncogenes (*Myc* and *Bcl-2*), the repression of inflammatory cytokines [interleukin (IL)-1, IL-8, TNF-α, and TGF-β], or no change [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and γ-actin] (17–27). HDAC inhibitors have exhibited properties in inducing cell-cycle arrest, cell differentiation, and apoptotic cell death in tumor cells and in decreasing inflammation and fibrosis in inflammatory diseases (17–32). Although the effects of HDAC inhibitors induce bulk histone acetylation, they result in apoptotic cell death, terminal differentiation, and growth arrest in tumor cells but no toxicity in normal cells (17, 21, 22, 33). In addition, the modulation of chromatin conformation by HDAC inhibitors can further radiosensitize tumors, the cells of which are intrinsically radioresistant (34–36).

Thus, on the basis of the potential possibility in simultaneously, coordinately, selectively, and epigenetically manipulating the expression of tumor suppressors, oncogenes, proinflammatory cytokines (TNF-α), and fibrogenic growth factors (TGF-β) by differentially remodeling the chromatins in normal and tumor cells, the use of HDAC

inhibitors as topical agents to prevent systemic reactions warrants study, to test whether they can decrease radiation-induced skin damage and still exhibit antitumor effects. The present study may provide a novel strategy to maximize the therapeutic effectiveness of cancer radiotherapy.

Materials and Methods

Topical Drug Formulations and Preparation

The preparation of the topical HDAC inhibitors has been described elsewhere (37). In brief, different ratios of various amounts of sodium phenylbutyrate (Triple Crown America, Perkasie, PA), valproic acid (Sigma Chemical Co., St. Louis, MO) or trichostatin A (Sigma), white petrolatum, cetyl alcohol, paraffin, tefose, superpolystate, methylparaben, propylparaben, deionized water, Coster 5000, and Myriyol (all from Merck & Co., Whitehouse Station, NJ) were mixed, stirred at 400 rpm for 5 min at 70°C to form a paste, and then cooled at room temperature. Tests for skin permeation (using skin purchased from Ohio Valley Tissue & Skin Center, Cincinnati, OH), drug stability, skin irritation, and shelf life were performed at the Drug Delivery Department, Biochemical Engineering Center, Industrial Technology Research Institute, Hsinchu, Taiwan. Only the formulations with good stability, high skin penetration, low skin irritation, and long shelf life were selected for the study.

Generation of CRS, Treatment, and Evaluation of Skin Reactions

Skin over the gluteal area as large as 2 cm × 2 cm of adult female Sprague-Dawley rats weighing 150–175 g were irradiated by an electron beam with 6 MeV of energy on day 0 at 4 Gy/min up to 40 Gy to the prepared area after the rats were anesthetized. Vehicle, Vaseline (a negative control), madecassol (a positive control), or different HDAC inhibitors were applied topically. at a dose of 200 mg/irradiated skin surface twice per day from Day 1 to Day 90. Skin reactions were evaluated and scored through 90 days after irradiation using the modified skin score system proposed elsewhere as follows: 0, normal; 0.5, slight epilation; 1.0, epilation in about 50% of the radiated area; 1.5, epilation in more than 50% of the area; 2.0, complete epilation; 2.5, dry desquamation in more than 50% of the area; 3.0, moist desquamation in a small area; and 3.5, moist desquamation in most of the area. Each point represents the mean of skin scores from five samples in the same group (38). Irradiated skins were subjected to RNase protection assays at the indicated times. After 90 days, skin reactions were evaluated by hematoxylin and eosin (H&E) histological testing, immunohistochemistry, and immunofluorescence. All experimental and surgical procedures performed on rats and mice were in accordance with the NIH guidelines outlined in the *Guide for Care and Use of Laboratory Animals* (NIH publication 85-23).

RNase Protection Assay

Levels of TGF-β1, TGF-β2, TGF-β3, and TNF-α mRNA were assessed using a multiple cytokine RNase protection assay kit (Riboquant; PharMingen, San Diego, CA)

that contained a template set to allow for the generation of a ^{32}P-labeled antisense RNA probe set that hybridized with the target mRNA for TGF-β1, TGF-β2, TGF-β3, TNF-α, and internal control GAPDH. After hybridization of labeled probe to target RNA, unprotected RNA was digested by an RNase, and protected RNA fragments were resolved on a 6% polyacrylamide gel and recorded by phosphorimaging (Molecular Dynamics Corp., Sunnyvale, CA). Densitometry was used to quantify the amount of each mRNA species and was normalized to the internal control GAPDH.

Statistical Analysis

The means of skin scores for skin reactions from five rats in each group were calculated. The average levels of cytokine/growth factor mRNA from three skin samples in each group were normalized to the internal control GAPDH and expressed as a ratio to the average level in time-matched control groups. The Mann-Whitney test (Stata Statistical Software, College Station, TX) was used to determine statistical significance at the $P < 0.05$ level for differences in average skin scores and in average mRNA levels, respectively, between treated and control rats.

Histological, Immunohistochemical, and Immunofluorescence Tests

The samples were fixed in 10% formalin-buffered solution and embedded in paraffin wax. Serial 3-μm sections were cut, dewaxed, and stained with H&E-safranin. For immunohistochemical analyses, the paraffin sections were deparaffinized by xylene and rehydrated by sequential concentrated alcohols. The slides were subjected to microwave antigen retrieval (800 W, twice for 5 min each) in 0.01 m sodium citrate buffer (pH 6.0). The endogenous hydrogen peroxidase activity was blocked by 3% H_2O_2 for 10 min. The slides were incubated with a protein-blocking agent (Dako, Glostrup, Denmark) for 20 min and then treated with the rabbit polyclonal anti-TNF-α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, followed by the secondary anti-rabbit antibody incubation for 30 min, and then they were washed with PBS three times. The peroxidase reaction was visualized with AEC (3% 3-amino-9-ethylcarbazole in N,N-dimethylformamide) as chromogen for 3–10 min, and then the slides were counterstained with Mayer's hematoxylin and mounted. Because inflamed skin tends to bind antibodies nonspecifically, we also used an isotype-matched normal antibody as a control, to confirm positive TNF-α staining in the wound. For immunofluorescence analyses, the paraffin sections were treated using the same protocol as those for immunohistochemical testing, except for the use of primary goat anti-acetylated histone H3 and rabbit anti-TGF-β1 and TGF-β2 antibodies (all from Santa Cruz Biotechnology), and the secondary rhodamine-conjugated (red) anti-goat (Jackson ImmunoResearch, West Grove, PA) and FITC-labeled (green) anti-rabbit antibodies (Dako).

Northern Blot Assay

Total RNA was isolated from frozen skin samples using Trigent (Molecular Research Center Inc., Cincinnati, OH). Total RNA (30 μg) was electrophoresed in a

denaturing formaldehyde-agarose gel, blotted onto Hybond N (Amersham, Amersham, United Kingdom), and fixed by UV irradiation. The membrane was incubated with ^{32}P-labeled probes, as described below, in Rapid-hyb buffer (Amersham). To prepare probes for rat TGF-β1 and TGF-β2, full-length coding sequences were amplified by reverse-transcription PCR using specific forward (TGF-β1, 5'-CGGGTGGCAGGCGAGAGC-3' and TGF-β2, 5'-CATGCACTACTGTGTGCT-3') and reverse (TGF-β1, 5'-GGAATTGTTGCTATATTTCTGC-3' and TGF-β2, 5'-CCGAGGACTTTAGCTGCA-3') primers. A template set of TNF-α and GAPDH from the RNase protection assay kit (Riboquant; PharMingen) was used to generate ^{32}P-labeled antisense RNA probes that hybridized with the mRNA for TNF-α and GAPDH.

Western Blot Assay

For the analysis of acetylated histones, collected irradiated skins, treated with or without phenylbutyrate were digested with 5 mg/ml collagenase (Sigma) and 1.5 mg/ml DNase (Sigma) and were passed through a wire mesh to prepare isolated cells. Nuclei were then isolated by lysis of the cells in a buffer that contained 10 mM Tris-HCl (pH 6.5), 50 mM sodium bisulfite, 1% Triton X-100, 10 mM MgCl$_2$, and 8.6% sucrose and a Dounce homogenizer was used. Histones were isolated by means of acid extraction, as described elsewhere (39). Isolated histones (5 μg) were then separated in 15% polyacrylamide-0.1% SDS minigels (Bio-Rad, Hercules, CA) and transferred to nitrocellular filters. The acetylated histones H3 were detected with antiacetylated histone H3 (Santa Cruz Biotechnology). For analysis of p21^{Cip1} in the cultured tumor cells treated with or without phenylbutyrate, cells were rinsed twice with PBS without Mg^{2+} and Ca^{2+} [PBS(−)] and lysed with PBS(−) supplemented with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 3 trypsin inhibitor units/ml aprotinin, and 1 mM sodium orthovanadate (Sigma). The lysate was gently rotated for 60 min at 4°C and centrifuged at 15,000 × g for 30 min at 4°C. The supernatant, which contained 100 μg of proteins, was electrophoresed in 10% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The amount of p21^{Cip1} was detected with the primary mouse monoclonal antibody p21^{Cip1} (Santa Cruz Biotechnology) for 16 h at 4°C. The membranes were then incubated with the peroxidase-labeled secondary anti-mouse antibody (Amersham) for 1 h at room temperature. The membranes were rinsed, treated with enhanced chemiluminescent reagent (Amersham), and exposed to films.

Generation of Cutaneous Tumor Models and Treatment

The syngeneic carcinoma cells 1MEA7R.1 and CT-26 (purchased from American Type Tissue Culture Collection, Manassas, VA) were s.c. injected into the flank areas of female BALB/c mice. The tumor was allowed to a maximum dimension of 0.5 cm. Topical HDAC inhibitors or vehicle were applied at a dose of 200 mg/mouse to cover the whole tumor surface and surrounding skin twice per day for 4 weeks. The tumor size was calculated weekly with a caliper according to the formula $ab^2/2$, where a and b are the larger and smaller diameters (in centimeters), respectively.

Results

Topical Phenylbutyrate Treatment Induces Histone Hyperacetylation

Among the known HDAC inhibitors, phenylbutyrate, which is a natural low-toxic aromatic fatty acid purified from mammalian urine and plasma, has been approved by the US Food and Drug Administration for the treatment of inherited genetic diseases and malignancies (40–44). We prepared several topical formulations of phenylbutyrate to increase local concentrations, because a plasma level of phenylbutyrate above 0.5 mM (the minimal dose to induce histone hyperacetylation and antitumor effects) is difficult to maintain via the oral or i.v. routes (37, 45, 46). Among the different formulations, the phenylbutyrate cream (Tric-02-3), which showed good stability, low rates of skin irritation, a long shelf life, and a high rate of skin penetration, was selected for the present study (37) (Fig. 1A). To determine what dosage of topical phenylbutyrate was suitable to treat irradiated skin, we used the amount of histone hyperacetylation in the nucleus as a marker to demonstrate the extent of drug penetration and to indicate whether the local drug concentration was enough to exert biological effects. Western blot analysis for acetylated histones in the irradiated skin 6 h after irradiation (40 Gy single fraction) showed that the acetylated form of histone H3 was mildly increased in the control and vehicle-treated groups but was markedly increased with the topical treatment of 1% phenylbutyrate cream at a dose of 200 mg/irradiated skin surface given immediately after irradiation (Fig. 1B). Immunofluorescence staining further demonstrated that histone hyperacetylation in irradiated skin was visually evident deep in the s.c. layer at 6 h after drug treatment (i.e., coincident with the peak of the drug concentration in the skin test) (Fig. 1, A and C).

Topical Phenylbutyrate Treatment Suppresses CRS

The acute skin reaction after irradiation was scored to grade toxicity in five animals in each group receiving vehicle, phenylbutyrate, madecassol (a steroid-positive control), or Vaseline (a negative control) from Day 1 to Day 90 (Fig. 2). The skin score increased with a more severe skin reaction. The skin reactions in the phenylbutyrate group tended to be less marked than those in other groups. Transient skin erythema was noted within hours to the first 2 days in all groups. Depilation of the epidermis appeared earlier (at Day 5) in the phenylbutyrate group, but the scores in the Vaseline and vehicle control groups were higher within 11 days. By Day 21, the Vaseline and vehicle groups had progressed to wet desquamation in most areas, whereas in the madecassol and phenylbutyrate groups, wound healing in the epithelium had begun quickly.

After 90 days, the long-term effects of radiation in the skin were evaluated by histological testing. At Day 180, skin samples taken from the phenylbutyrate group showed
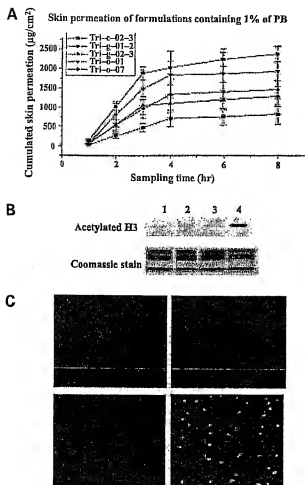
A

B

C

Figure 1. Induction of histone hyperacetylation in skin. A, pharmacokinetic studies of delivery of different phenylbutyrate formulations through the skin. Among them, the 1% phenylbutyrate (*PB*) cream (*Tri-c-02-3*) was used because of its stability and long shelf life. B, Western blot analysis for acetylated H3 in the irradiated skin (40 Gy single fraction) treated with or without phenylbutyrate cream for 6 h after irradiation. The Coomassie blue-stained gel sections demonstrates the equivalence of protein loading. *Lane 1,* normal skin without irradiation; *lane 2,* irradiated skin without any treatment; *lane 3,* irradiated skin treated with the vehicle; *lane 4,* irradiated skin treated with 1% phenylbutyrate cream. C, immunofluorescence staining of acetylated H3 in the irradiated skin treated with or without phenylbutyrate cream for 6 h after irradiation. The acetylated H3 in the nuclei was used as marker indicating the extent of drug penetration. *Left upper panel,* irradiated skin without any treatment; *right upper panel,* normal skin without irradiation; *left lower panel,* irradiated skin treated with 1% phenylbutyrate cream; end *right lower panel,* irradiated skin treated with the vehicle.

soft characteristics and less capillary bleeding, whereas skin samples taken from the vehicle group exhibited rigid characters and easily oozing. Histological sections were examined to provide a qualitative description of group differences in reepithelialization after irradiation and in the development of the dermal fibrosis (Fig. 3). The histology in the phenylbutyrate group at Day 180 showed a thicker epidermis with 10–30 cell layers, less subepithelial swelling, a thinner dermis with less collagen deposition, and few skin appendages, compared with the histological results in

the vehicle group at Day 180 and with the control groups of normal skin and acute reaction at Day 7. These observations suggest that phenylbutyrate promotes epithelial healing in early radiation-induced skin damage and inhibits late, radiation-related proliferative dermal fibrosis.

Changes in Radiation-Induced Histology after Topical Phenylbutyrate Treatment Correlate with Suppression in Inflammatory and Fibrogenic Cytokine Expressions

Because the development of CRS has been attributed to radiation-induced temporal changes and the persistent up-regulation of proinflammatory cytokines such as TNF-α and fibrogenic growth factors such as TGF-β1 and β2(3–16), we next studied whether the suppression of CRS development by the topical phenylbutyrate treatment is correlated with the suppression of TNF-α and TGF-β.

The timing of the peak appearance of TGF-β1, TGF-β2, and TNF-α expression levels induced by radiation correlated with the progression in CRS development in all experimental groups (Figs. 2–Figs. 4). In the phenylbutyrate group, the highest surge of TGF-β1, TGF-β2, and TNF-α appeared at 6 h after irradiation, but these levels were subsequently suppressed after Day 14. The suppression still persisted at 12 months, even when topical phenylbutyrate treatment was discontinued at Day 90. In the Vaseline and vehicle control groups, mRNA levels of TGF-β1, TGF-β2, and TNF-α in the irradiated skin increased and fluctuated above the nonirradiated control levels over a period of 1 year and reached the first peak of 2- and 3-fold above the nonirradiated control levels at 6 h after irradiation, the second peak of 10.5- to 16-fold around 14–28 days after irradiation, and the third peak of 13- to 14-fold at 9 months after irradiation; levels then declined to 2- to 3-fold normal levels by 12 months after irradiation. Although the mRNA levels of TGF-β1, TGF-β2, and TNF-α at the first peak at 6 h in the phenylbutyrate group were higher than those in the Vaseline and vehicle control groups, they decreased to the levels lower than those in the Vaseline and vehicle control groups at Day 14 and returned to the nonirradiated control levels at Day 28–35.

TGF-β1 and TGF-β2 have similar cellular effects in inhibiting epithelial cell growth and promoting dermal
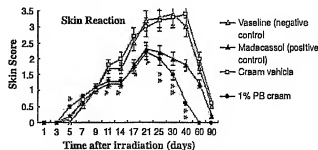

Figure 2. Evaluation of acute skin reactions after irradiation. Time course of the average skin score for the acute skin reaction after irradiation from Day 1 to Day 90. The skin score increases with more severe skin reaction. *, $P < 0.05$; **, $P < 0.001$, in comparison with the phenylbutyrate (*PB*)-treated and vehicle groups.
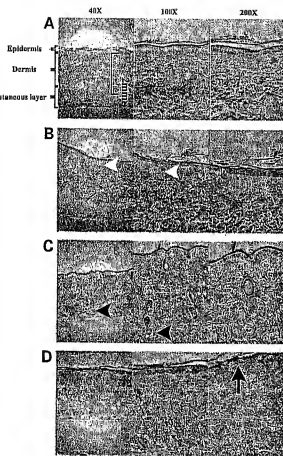
Figure 3. Histology of reaction after irradiation. Histological analysis by H&E stain of the skin damage. The histology in the phenylbutyrate-treated group at Day 180 (D) after skin irradiation showed thicker epidermis with 10–30 cell layers (*black arrow*), less subepithelial swelling, a thinner dermis with less collagen deposition, and few skin appendages compared with the histology in the vehicle group at Day 180 (C) and the control groups of normal skin at Day 0 (A) and acute reaction at Day 7 (B). The *black arrowheads* indicate that the s.c. fat layer in the vehicle group (C) was replaced by fibrous tissues and appendages. The *white arrowheads* indicate subepithelial swelling in the acute reaction group at Day 7 (B).

fibroblast proliferation, but TGF-β3 has the opposite effect (4, 10). Thus, we also studied the level change of TGF-β3 mRNA and found that it exhibited a slightly transient increase of 2-fold in all irradiated groups at 14 days then progressively decrease to the nonirradiated control level or lower. No significant differences in TGF-β3 mRNA levels were observed between the irradiated groups treated with Vaseline, vehicle, or phenylbutyrate.

Immunofluorescence staining further confirmed that TGF-β1 and β2 proteins were predominantly present in the keratinocytes of the thinner epidermis and in myofibroblasts of the proliferative thicker dermis in the vehicle group, whereas the amount of TGF-β1 and β2 protein was low in both the thicker epidermis and the thinner dermis in the phenylbutyrate group, compared with the staining pattern in normal skin (Fig. 5).

Moreover, at Day 270, three of five rats in the Vaseline group and four of five rats in the vehicle group, compared with zero of five rats in the phenylbutyrate-treated group, showed chronic ulceration, necrosis, bullae formation, and inflammatory cell infiltration. The decrease in late radiation-induced skin damage by topical phenylbutyrate was consistent with the suppression of TNF-α expression (Fig. 6).

Topical Phenylbutyrate Treatment Both Prevents Late Radiation-Induced Skin Tumor Formation and Exhibits a Direct Antitumor Effect on Cutaneous Tumor Growth

Many studies have demonstrated that the chronic overexpression of TGF-β stimulates neoplastic growth (8, 15). Thus, the decrease of TGF-β expression caused by phenylbutyrate treatment might decrease the incidence of late radiation-induced tumorigenesis. We found that newly developed skin or cutaneous tumors increased with time after 50 weeks in the control group that did not receive phenylbutyrate treatment, but no tumors developed in the phenylbutyrate-treated group (Fig. 7). At 90 weeks, a cumulative tumor incident of 15% (6 of 39) was observed in the irradiated group without phenylbutyrate treatment, compared with 0% (0 of 42) in the irradiated groups with phenylbutyrate treatment. The histology of radiation-induced tumors included fibroma, spindle cell carcoma, basal cell carcinoma, and squamous cell carcinoma.

The topical phenylbutyrate formulation was further tested to assess whether it retained its antitumor effects. BNL 1MEA7R.1 and CT-26 carcinoma cells, which showed growth inhibition by phenylbutyrate *in vitro* through the upregulation of p21^Cip1 (Fig. 8A), were inoculated into the back of syngeneic mice. Cutaneous tumors were allowed to grow to the largest dimension 0.5 cm, and phenylbutyrate was topically applied to the tumor surface at 200 mg/mouse twice per day. By 4 weeks, the tumor sizes of 1MEA7R.1 and CT-26 carcinomas in the placebo groups were almost 6- and 1.6-fold larger than those in the phenylbutyrate-treated groups, respectively (Fig. 8B). Cutaneous tumors in the phenylbutyrate-treated groups grew slowly, without skin ulceration, whereas tumors in the control or placebo group grew rapidly and showed a necrotic appearance and skin ulceration (Fig. 8C). After 5 weeks of treatment, withdrawal of topical phenylbutyrate resulted in a loss of tumor growth inhibition, and these tumors then reached the same size as those in the control or placebo groups within 2 weeks.

Similar Effects of Other Structurally Unrelated Antitumor HDAC Inhibitors in Treating CRS

In addition to phenylbutyrate, other structurally unrelated antitumor HDAC inhibitors, such as trichostatin A (an antifungal agent) and valproic acid (an antiseizure agent), also ameliorated the development of CRS and decreased the radiation-induced TGF-β1, TGF-β2, and TNF-α expression (Fig. 9, A and B).

Discussion

Although previous studies have demonstrated that HDAC inhibitors have effects in modulating multiple genes to
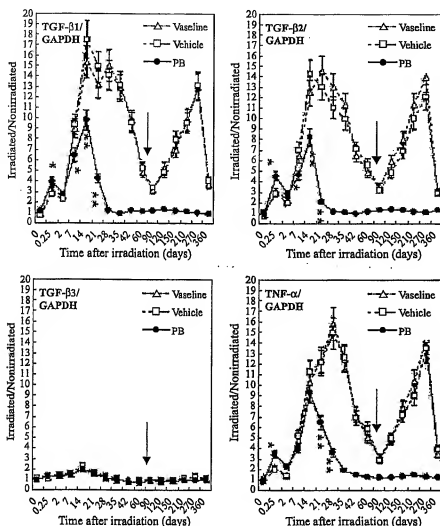
Figure 4. Expression levels of TGF-β and TNF-α after irradiation. Temporal variation in mRNA levels of TGF-β1, TGF-β2, TGF-β3, and TNF-α in skin after irradiation, normalized to the internal control GAPDH, and expressed as a ratio to levels in nonirradiated control samples. *Points*, mean of mRNA levels of five samples in the same group of Vaseline, vehicle, or phenylbutyrate (PB). The *arrow* indicates that the drug treatment was discontinued after Day 90 (*, $P < 0.05$; **, $P < 0.001$, in comparison with the phenylbutyrate-treated and vehicle groups).

inhibit tumor growth, reducing lipopolysaccharide induced circulating cytokines, affecting cytokine production to attenuate concanavalin A-induced hepatic injury, and reversing skewed cytokine expression in autoimmune inflammatory diseases (17, 18, 24, 26, 27), the present study appears to be the first to examine the cytokine- or gene-modulatory effects of the inhibition of HDAC in CRS and radiation-induced tumorigenesis. By virtue of reducing the levels of radiation-induced cytokines such as TNF-α and TGF-β, we found that HDAC inhibitors could be candidates

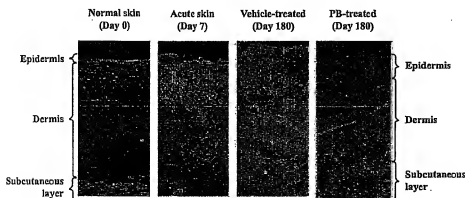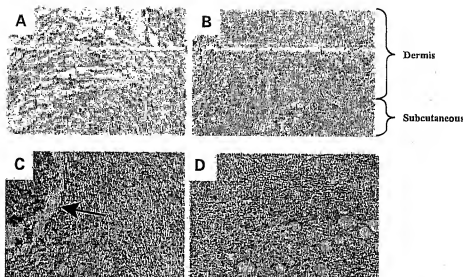Figure 5. Immunofluorescence. TGF-β expression in the skin after irradiation. The expression level of TGF-β in the irradiated skin was high in the superficial dermis of acute reaction at Day 7 and in the thin epidermis and proliferative dermis in the vehicle group at Day 180 but was low in the hyperplastic epidermis and thinner dermis in the phenylbutyrate (PB) group at Day 180.

Figure 6. Immunohistochemistry. TNF-α expression in the long-term skin damage at Day 270 after irradiation. The expression levels of TNF-α in the normal skin and phenylbutyrate-treated group were low. In contrast, the high expression levels of TNF-α in the Vaseline and vehicle groups correlated with skin necrosis and heavy inflammatory infiltrates. A, normal skin; B, irradiated skin treated with the topical phenylbutyrate; C, irradiated skin treated with Vaseline (the *arrow* indicates the necrotic wound); and D, irradiated skin treated with vehicle.

for the treatment of CRS and the prevention of late tumorigenesis. Although the down-regulation of TNF-α and TGF-β by the inhibition of HDAC is likely to contribute to the effects observed in the present study, HDAC inhibitors may have another mechanisms, for example, the hyperacetylation of nonhistone proteins such as ribosomal S3 or the Rel-A subunit of NF-κB (47, 48). Moreover, as a consequence of nuclear hyperacetylation, HDAC inhibitors also up-regulate the cell cycle kinase inhibitor $p21^{Cip1}$, which, in part, accounts for their antitumor properties (21).

TGF-β is a pleiotropic growth factor that inhibits epithelial cell growth but promotes mesenchymal cell proliferation and neoplastic growth (8–10, 15, 16). In
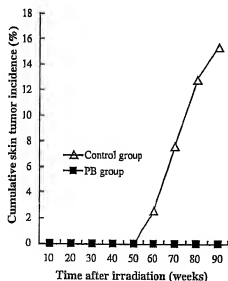

Figure 7. Skin tumorigenesis after irradiation. Cumulative skin tumor incidence after skin irradiation increased with time in the groups without the topical phenylbutyrate (*PB*) treatment.
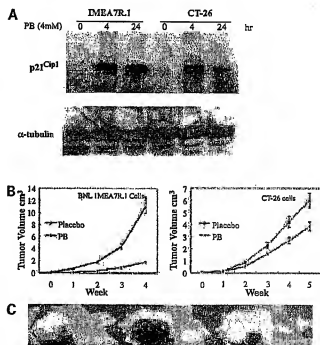

Figure 8. Phenylbutyrate inhibits tumor growth. A, time-course analysis of the up-regulated levels of $p21^{Cip1}$ protein in BNL 1MEA7R.1 and CT-26 carcinoma cells during treatment with 4 mM phenylbutyrate (*PB*). B, growth inhibition test *in vivo*. The cutaneous tumors (*BNL 1MEA7R.1* and *CT-26* carcinoma cells), grown to a size of 0.5 cm, were treated with the topical phenylbutyrate or vehicle at a dose of 200 mg/mouse, to cover the whole tumor surface and surrounding skin twice per day for 4 weeks. At the end of this period, the cutaneous tumor sizes of 1MEA7R.1 and CT-26 in mice in the placebo groups were almost 6- and 1.6-fold larger than those in the topical phenylbutyrate-treated groups, respectively. C, an initial tumor size of 1MEA7R.1 beneath the skin about 0.5 cm in dimension before treatment (*left*), a placebo- or vehicle-treated tumor at week 4 (*middle*), and a phenylbutyrate-treated tumor at week 4 (*right*).
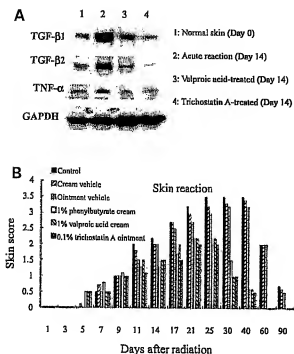
Figure 9. Effects of other structurally unrelated HDAC inhibitors in CRS. The different HDAC inhibitors (trichostatin A and valproic acid) also suppressed the radiation-induced levels of TGF-β1, TGF-β2, and TNF-α expression and ameliorated CRS. A, Northern blot assays for TGF-β1, TGF-β2, and TNF-α expressions. B, skin scores for skin reactions after radiation. The skin score increases with a more severe skin reaction.

the present study, the sustained high expression levels of TGF-β1 and -β2 in irradiated skin was significant in explaining the manifestation of thin epidermis and proliferative dermis in the vehicle group at Day 180 as well as the occurrence of tumor formation after 50 weeks. In contrast, the appearance of hyperplastic epidermis and a thinner dermis at Week 90 after irradiation in the phenylbutyrate-treated group, correlated with the early suppression of the chronic up-regulation of TGF-β1 and -β2 by phenylbutyrate. Although TGF-β is certainly a key growth factor, the development of CRS and the recovery from a perpetual wound might not depend on the modulation of a single factor. The wound healing process after irradiation involves a complex feedback network of interacting cytokines and growth factors, including platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor, granulocyte macrophage colony-stimulating factor, connective tissue growth factor, IL-1, IL-4, inhibiting growth factor-1, and TNF-α, and complex interactions among the parenchymal, epithelial, endothelial, and inflammatory cells (3, 12). Among them, TNF-α is implicated in triggering the recruitment of inflammatory cells through the expression of adhesion molecules on the vascular surface, as well as the stimulation of fibroblast proliferation (12, 49, 50). Thus, in addition to the down-regulation of long-term TGF-β activation, the simul-

taneous suppression of radiation-induced TNF-α in mesenchymal cells and macrophages should also contribute to the decrease in acute skin inflammation and the incidence of late skin necrosis and fibrosis seen in the present study.

Drugs that have previously been tested in the management of CRS include antioxidants (vitamin E and superoxide dismutase), anti-inflammatory agents (corticosteroids, colchicines, D-penicillamine, and TNF-α antagonist antibodies), and anti-fibrogenic agents (IFN, TGF-β antagonist, and angiotensin-converting enzyme inhibitors) (7). However, few of these are able to simultaneously ameliorate acute dermatitis, prevent the occurrence of fibrosis, and reduce late tumorigenesis; moreover, toxicities, side effects, tumor protection possibilities, and a lack of antitumor effects are troublesome. Rather than focusing on agents that act only on a few steps in the pathogenesis of CRS, our study provides a novel strategy to exert anti-inflammatory, anti-fibrogenic, and antitumor effects at one time by HDAC inhibitors to remodel chromatins and epigenetically regulate multiple genes involved in radiation-induced skin damage and tumor growth.

To our knowledge, this is the first time that the antitumor HDAC inhibitors have been shown to protect skin from radiation-induced damage and tumorigenesis. Our study warrants further clinical investigations into the combination of therapeutic radiation and HDAC inhibitors in increasing the therapeutic gain of cancer radiotherapy. Although TGF-β and TNF-α could be the targets of HDAC inhibitors, more cellular and molecular studies are needed to identify the precise mechanisms of HDAC inhibitors in both suppressing CRS and preventing tumorigenesis.

References

1. Peter RU. The cutaneous radiation syndrome. Advances in the treatment of radiation injuries. Oxford: Elsevier; 1998. p. 237–40.

2. Hopewell JW. The skin: its structure and response to ionizing radiation. Int J Radiat Biol. 1990;57:751–73.

3. Hill RP, Rodemann HP, Hendry JH, Roberts SA, Anscher MS. Normal tissue radiobiology: from the laboratory to the clinic. Int J Radiat Oncol Biol Phys. 2001;49:53–65.

4. Seong J, Kim SH, Chung EJ, Lee WJ, Suh CO. Early alteration in TGF-β mRNA expression in irradiated rat liver. Int J Radiat Oncol Biol Phys. 2000;46:639–43.

5. Wang J, Zheng H, Sung CC, Richter KK, Hauer-Jensen M. Cellular sources of transforming growth factor-β isoforms in early and chronic radiation enteropathy. Am J Pathol. 1998;153:1531–40.

6. Fedorocko P, Egyed A, Vacek A. Irradiation induces increased production of haemopoietic and proinflammatory cytokines in the mouse lung. Int J Radiat Biol. 2002;78:305–13.

7. Martin M, Lefaix J, Delanian S. TGF-β1 and radiation fibrosis: a master switch and a specific therapeutic target. Int J Radiat Oncol Biol Phys. 2000;47:277–90.

8. Randall K, Coggle JE. Long-term expression of transforming growth factor β1 in mouse skin after localized β-irradiation. Int J Radiat Biol. 1996;70:351–60.

9. Border WA, Noble NA. Transforming growth factor β in tissue fibrosis. N Engl J Med. 1994;331:1286–92.

10. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med. 1999; 341:738–46.

11. Zhou D, Yu T, Chen G, et al. Effects of NF-κB (p50) targeted gene disruption on ionizing radiation-induced NF-κB activation and TNFα, IL-1α, IL-1β and IL-6 mRNA expression in vivo. Int J Radiat Biol. 2001;77:763–72.

12. Skwarchuk MW, Travis EL. Changes in histology and fibrogenic cytokines in irradiated colorectum of two murine strains. Int J Radiat Oncol Biol Phys, 1998;42:169 – 78.

13. Siven V, Vozenin-Brotons MC, Tricaud Y, et al. Altered proliferation and differentiation of human epidermis in cases of skin fibrosis after radiotherapy. Int J Radiat Oncol Biol Phys, 2002;53:385 – 93.

14. Delanian S, Martin M, Bravard A, Luccloni C, Lefaix JL. Abnormal phenotype of cultured fibroblasts in human skin with chronic radiotherapy damage. Radiother Oncol, 1998;47:255 – 61.

15. Wieser R. The transforming growth factor-β signaling pathway in tumorigenesis. Curr Opin Oncol, 2001;13:70 – 7.

16. Massague J, Blain SW, Lo RS. TGFβ signaling in growth control, cancer, and heritable disorders. Cell, 2001;103:295 – 309.

17. Marks PA, Richon VM, Rifkind RA. Histone deacetylase inhibitors: induction of differentiation or apoptosis of transformed cells. J Natl Cancer Inst, 2000;92:1210 – 6.

18. Kramer OH, Gottlicher M, Heinzel T. Histone deacetylase as a therapeutic target. Trends Endocrinol Metab, 2001;12:294 – 300.

19. Leggar G, O'Carroll D, Rembold M, et al. Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J, 2002;21:2672 – 81.

20. Richon VM, O'Brian JP. Histone deacetylase inhibitors: a new class of potential therapeutic agents for cancer treatment. Clin Cancer Res, 2002; 8:662 – 7.

21. Richon VM, Sandhoff TW, Rifkind RA, Marks PA. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci USA, 2000;97:10014 – 9.

22. Van Lint C, Emiliani S, Verdin, E. The expansion of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Exp, 1996;5:245 – 53.

23. Huang N, Katz JP, Martin DR, Wu GD. Inhibition of IL-8 gene expression in Caco-2 cells by compounds which induce histone hyper-acetylation. Cytokine, 1997;9:27 – 36.

24. Mishra N, Brown DR, Olorenshaw IM, Kammer GM. TSA reverses skewed expression of CD154, interleukin-10, and interferon-γ gene and protein expression in lupus T cells. Proc Natl Acad Sci USA, 2001;98: 2628 – 33.

25. Stockhammer G, Manley GT, Johnson R, Rosenblum MK, Samid D, Lieberman RS. Inhibition of proliferation and induction of differentiation in medulloblastoma- and astrocytoma-derived cell lines with phenylacetate. J Neurosurg, 1995;83:672 – 81.

26. Sagain JP, Reingeard de la Blatiere D, Bourrelie A, et al. Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease. Gut, 2000;47:397 – 403.

27. Leoni F, Zaliani A, Bertolini G, et al. The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. Proc Natl Acad Sci USA, 2002;99: 2995 – 3000.

28. Warrell RP Jr, Ha LZ, Richon V, Calleja E, Pendolfi PP. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. J Natl Cancer Inst, 1998;90:1621 – 5.

29. Vigushin DM, Ali S, Pace PE, et al. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo. Clin Cancer Res, 2001;7:971 – 6.

30. Saunders N, Dicker A, Popa C, Jones S, Dahler A. Histone deacetylase inhibitors as potential anti-skin cancer agents. Cancer Res, 1999;59:399 – 404.

31. Gottlicher M, Minucci S, Zhu P, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J, 2001;20:6969 – 78.

32. Rombouts K, Niki T, Wielant A, Hallemans K, Geerts A. Trichostatin A, lead compound for development of antifibrogenic drugs. Acta Gastroenterol Belg, 2001;64:239 – 46.

33. Gerber K. Silence of the genes: cancer epigenetics arrives. J Natl Cancer Inst, 2002;94:793 – 5.

34. Ferrandina G, Filippini F, Ferlini C, et al. Growth inhibitory effects and radiosensitization induced by fatty aromatic acids on human cervical cancer cells. Oncol Res, 2001;12:429 – 40.

35. Miller AC, Whittaker T, Thibault A, Samid D. Modulation of radiation response of human tumour cells by the differentiation inducers, phenyl-acetate and phenylbutyrate. Int J Radiat Biol, 1997;72:211 – 8.

36. Blade S, Stobbe CC, Boyd JT, Chapman JD. Chemical agents that promote chromatin compaction radiosensitize tumour cells. Int J Radiat Biol, 2001;77:1033 – 42.

37. Chung YL, Yen RL, Wang AJ, Yeo LF. Treating radiation fibrosis. United States Patent 6,538,030. 2003.

38. Abe Y, Urano M. Fraction size-dependent acute skin reaction of mice after multiple twice-a-dey doses. Int J Radiat Oncol Biol Phys, 1990;18: 359 – 64.

39. Richon VM, Emiliani S, Verdin E, et al. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci USA, 1998;95:3003 – 7.

40. Brusilow SW, Danney M, Waber LJ, et al. Treatment of episodic hyperammonemie in children with inborn errors of urea synthesis. N Engl J Med, 1984;310:1630 – 4.

41. Olivieri NF, Rees DC, Ginder GD, et al. Treatment of thalassaemia major with phenylbutyrate and hydroxyurea. Lancet, 1997;350:491 – 2.

42. Rubenstein RC, Zeitlin PL. A pilot clinical trial of oral sodium 4-phenylbutyrate (Buphenyl) in ΔF508-homozygous cystic fibrosis patients: partial restoration of nasal epithelial CFTR function. Am J Respir Crit Care Med, 1998;157:484 – 90.

43. Kemp S, Wei HM, Lu JF, et al. Gene redundancy and pharmacological gene therapy: implications for X-linked adrenoleukodystrophy. Nat Med, 1998;4:1261 – 8.

44. Gore SD, Weng LJ, Zhai S, et al. Impact of the putative differentiating agent sodium phenylbutyrate on myelodysplastic syndromes and acute myeloid leukemia. Clin Cancer Res, 2001;7:2330 – 9.

45. Gilbert J, Baker SD, Bowling MK, et al. A phase I dose escalation and bioavailability study of oral sodium phenylbutyrate in patients with refractory solid tumor malignancies. Clin Cancer Res, 2001;7: 2292 – 300.

46. Carducci MA, Gilbert J, Bowling MK, et al. A phase I clinical and pharmacological evaluation of sodium phenylbutyrate on a 120-h infusion schedule. Clin Cancer Res, 2001;7:3047 – 55.

47. Webb Y, Zhou X, Ngo L, et al. Photoaffinity labeling and mass spectrometry identify ribosomal protein S3 as a potential target for hybrid polar cytodifferentiation agents. J Biol Chem, 1999;274:14280 – 7.

48. Chen L, Fischle W, Verdin E, Greene WC. Duration of nuclear NF-κB action regulated by reversible acetylation. Science, 2001;293:1653 – 7.

49. Zhang K, Phan SH. Cytokines and pulmonary fibrosis. Biol Signals, 1996;5:232 – 9.

50. Van Deventer SJ. Tumor necrosis factor and Crohn's disease. Gut, 1997;40:443 – 7.